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A STUDY OF THE RELATIVE EFFICIENCY OF THE VARIOUS DIFFERENTIAL STAINING METHODS USED IN IDENTIFYING THE TUBERCLE BACILLUS, ... *N. P. Sherwood.*

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A Study of the Relative Efficiency of the Various Differential Staining Methods Used in Identifying the Tubercle Bacillus.*

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THERE has been much discussion as to the classification of the tubercle, leprosy, smegma, grass, and butter bacilli. Migula classifies them with the Bactereaceæ, while Abbot and others have placed them among the trichobacteria. They differ from the rest of the bacteria in that they contain fats, waxes and alcohols, which render them quite difficult to stain with the ordinary basic anilin dyes, such as methylene blue, basic fuchsin, or gentian violet. By covering a dried and fixed preparation of these bacteria on a slide with basic fuchsin or gentian violet, and steaming it from three to five minutes over a free flame, the stain is taken up and retained by these organisms with much greater tenacity than practically any other members of the bacterial kingdom. In fact, when fuchsin is steamed into them they resist decolorization by three to five per cent aqueous solution of any of the mineral acids for thirty minutes to an hour or even longer, whereas other bacteria are decolorized by this treatment. For this reason the tubercle, leprosy, smegma, grass, and butter bacilli are called the acid-fast group of bacteria. Jordan[1] says that this property is due largely to the presence of mykol, one of the higher alcohols. Spores of such organisms as *Bacillus subtilus* and *Bacillus anthracis* are also acid-fast, although the respective organisms are not.

This property of resistance to decolorization with mineral acids, when once the organisms have the stain steamed into them, has

* Read before Kansas Academy of Science January, 1916.

been taken advantage of by bacteriologists and made use of in the routine laboratory examination of sputum, urine, pus, etc., for tubercle bacilli in suspected cases of tuberculosis, and by many in examining butter, milk and other foods for the presence of tubercle bacilli. Now, as was stated above, this acid-fastness or acid-proofness is not the property of just one organism but of a small group of organisms. It has been the aim of many workers to find some method of decolorization that would enable one to differentiate between true tubercle bacilli and other members of this group. This seems to be desirable in order to eliminate possibilities of error in the laboratory examination of suspected tuberculous material, since the smegma bacillus is very commonly found in normal smegma, and has been reported as found, occasionally, in gangrenous conditions of the lungs, on the tonsils, in the tartar from the teeth, from scrapings from the tongue, and on the skin in the inguinal and axillary regions. The butter bacillus and grass bacillus may be present in butter, in other dairy products, and foods. Hence, either of these last two organisms may be found present in the mouths of normal individuals. Since tuberculosis is caused only by the tubercle bacillus and not by any other members of the acid-fast group, it is desirable when examining suspected material to rule out possibilities of error due to the presence of these other organisms. Many staining methods have been suggested, tried and recommended. They may be grouped into five groups as to theories and methods:

1. That true tubercle bacilli differ from the rest of the acid-fast group in their resistance to decolorization with 25 or 30 per cent aqueous solution of the mineral acids. All of the acid-fast organisms, excepting true tubercle bacilli, are said to be decolorized.

2. That while this group is acid-fast, only true tubercle bacilli are acid-alcohol fast. Hence, acidulated alcohol (2 to 3 per cent hydrochloric or nitric acids in from 70 per cent to absolute alcohol) has been used as a differential agent.

3. Some modification of (2), such as Pappenheim's, where rosalic acid is used in connection with glycerine and absolute alcohol.

4. That it is necessary and important to remove the fats and use strong concentrations of mineral acids and absolute alcohol to decolorize everything except the tubercle bacillus.

5. Fonte's method, which is a combination of the acid-fast and Gram's stain, as described below.

As a method typifying the first theory, Gabbet's[2] is perhaps the best. In this method the preparation is made and stained with steaming carbol-fuchsin from three to five minutes. The excess of staining fluid is drained off without washing and is replaced by Gabbet's methylene blue solution (2 gr. M. B.; sulphuric acid, 25 cc.; water, 75 cc.). This solution is allowed to act for from one to three minutes and is then washed off with water and the preparation dried and examined. The tubercle bacilli will appear as bright red rods, while all the other organisms are said to be stained blue.

The Ziehl-Neelsen[3] method comes under the second group. In this method the organisms are stained with steaming carbol-fuchsin from three to five minutes and decolorized with acidulated alcohol for several minutes, or until no more color comes from the slide. It is then washed in water and counter-stained with Loeffler's methylene blue. The tubercle bacillus will appear red, while all the other bacteria are supposed to be blue.

A modification of this method, which is reported to have given greater efficiency, is Pappenheim's[4] method. The preliminary staining is carried out as above. The specimen is then drained and covered with decolorizing solution, which is made by dissolving one gram of rosalic acid in 100 cc. of absolute alcohol, saturating the mixture with methylene blue and adding twenty parts of glycerine. The slide is then washed in water, dried between blotting paper, and examined with the immersion lens. The tubercle bacilli are stained red and all the other organisms are said to be blue.

As an example of group IV is Bunge and Trantenroth's[5] method. After fixation of the smear the fat is removed by soaking the specimens in absolute alcohol. The preparation is now covered with a 5 per cent solution of chromic acid for fifteen minutes, after which it is washed with water. The smear is stained with steaming carbol-fuchsin, decolorized with 16 per cent sulphuric acid for three minutes, and is then counter-stained for five minutes in a concentrated alcoholic solution of methylene blue. This method is said to give the tubercle bacillus a distinct red color, while the smegma bacillus is blue.

Fonte's[6] method for the differentiation of the tubercle bacillus from the rest of the acid-fast group is as follows:

1. Stain the dried and fixed preparation with steaming carbol-fuchsin for three minutes.
2. Wash with tap water.

3. Stain in the cold, with carbol-gentian violet, for two or three minutes.

4. Drain off the stain and treat with Lugol's solution until no more metallic mirrors appear. Blot dry with filter paper.

5. Decolorize with absolute alcohol and ether (equal parts) until no more color comes from the slide.

6. Counter-stain with methylene blue.

7. Wash in distilled water and dry, and examine.

The tubercle bacilli appear as bright red rods containing blue granules. The other members of the acid-fast group, as well as other Gram positive organisms, appear Gram positive.

The scope of this paper is an investigation of the value of Gabbet's, Ziehl-Neelsen's, Pappenheim's, Bunge and Trantenroth's, and Fonte's methods as a means of differentiating between *Bacillus tuberculosis, B. lepræ, B. smegma,* Moeller's grass bacillus, and the butter bacillus.

This work was undertaken because of the various conflicting statements in the literature as to the value of these solutions in differentiating between true tubercle bacilli and the rest of the acid-fast group; because Pappenheim's, Bunge and Trantenroth's, and Ziehl-Neelsen's methods are accepted and used in many laboratories as giving a reliable differentiation.

Alvarez and Travil[7] (1885), Klemperer and Bittu,[8] and Cowie[20] (1900-'01), as well as others, have described peculiar bacilli in smegma taken from the genitals of man and the lower animals, as well as from moist skin in the folds of the groin, the axilla and the arms. They also mention its presence in normal urine and sometimes in saliva and sputum. They are described as morphologically resembling the tubercle bacillus and resisting decolorization by mineral acids, but being decolorized by alcohol.

Moeller[9] (1898) found in milk, butter, timothy hay, cow dung, etc., acid-fast organisms. They were acid-alcohol-fast but nonpathogenic for laboratory animals. Moeller (1902) cultivated acid-fast organisms from the skin. He concluded that all acid-fast organisms that he had worked with were tinctorially like the true tubercle bacillus.

Marzinowski[10] (1900) found saprophytic acid-fast organisms associated with bronchitis. Frinkel[11] (1898), and Rabinovitch[13] (1900), found acid-fast organisms associated with pulmonary gangrene; they were saprophytes. Petri (1897), Rabinovitch[12] (1899) and Korn[14] (1899) have described as *Bacillus butyricus* an acid-fast organism, morphologically like the tubercle bacillus,

which may at times be found in butter. They think it would be mistaken for the tubercle bacillus from its morphology and staining reactions. It could be differentiated on cultural grounds, inasmuch as it grows readily on artificial media, whereas the tubercle bacillus does not.

Abbot and Gildersleeve[15] (1902) concluded from their study of the acid-fast group that 30 per cent nitric acid in water is a fairly satisfactory method of differentiation. They state that the majority of acid-fast bacteria are unable to resist decolorization with this nitric acid solution, whereas it does not affect the stain in the tubercle bacillus. They would also place the acid-fast organisms in the group of *Actinomycetes*. In regard to animal inoculation, they state that some of the acid-fast bacteria other than the tubercle bacillus are capable of causing in rabbits and guinea pigs nodular lesions suggestive of tubercles; that these lesions, while often very much like tubercles in their histological structure, may nevertheless be distinguished from them by the following peculiarities:

A. When occurring as the result of intravenous inoculation, they are always seen in the kidneys, only occasionally in the lungs, and practically not at all in the other organs.

B. They constitute a localized lesion, having no tendency to dissemination, metastasis, or progressive destruction of tissue, by caseation.

C. They tend to terminate in suppuration or organization rather than in progressive caseation as in the case with tubercles.

D. They are more commonly and conspicuously marked by the *Actinomycetes* type of development of the organisms than is the case with true tubercles, and these *Actinomycetes* are less resistant to decolorization by strong acid solutions than are those seen in tubercles.

For the experimental work of this paper, cultures of the various members of the acid-fast group were obtained from year to year, from the American Museum of Natural History. A culture of the tubercle bacillus was obtained from the University of Michigan and one from the University of Chicago. Two cultures (two strains) of the leprosy bacillus were obtained from Tulane University. Sputums from clinical cases of tuberculosis were also examined. In addition to the pure cultures of *B. smegmatis* obtained from the American Museum, normal smegma was secured from a number of individuals and used in these experiments.

Several samples of butter were also examined for the butter bacillus.

The pure cultures were carried along on glycerine agar, plain agar, and in glycerine broth. Cultures varying from one to three weeks in age were found satisfactory.

In studying the relative efficiency of Gabbet's, Pappenheim's, acid-alcohol, etc., as decolorizing agents the standard was the number of acid-fast organisms present when decolorized with 3 per cent nitric acid in water. Several slides were run on each of these as controls and in making estimates.

In the following table four plus (+ + + +) indicates complete acid-fastness with the reagent used, while a minus sign (—) indicates non-acid-fastness. The intermediate degrees are indicated by the number of plus signs, varying between the two limits. Where only a few organisms retain the stain, and that only to a slight degree, the double dagger is used (††).

DATA.

Source of Organism.	Staining and decolorizing methods—counter-stained with methylene blue.					
	3 per ct. nitric acid in water.	Gabbot's.	Pappenheim's.	Acid alcohol.	Bunge and Tranteroth's.	Fonte's.
B. tuberculosis.						
1. Cult., A. M., '12	++++	++++	++++	++++	++++	
2. Cult., A. M., '13	++++	++++	++++	++++	++++	
3. Cult., A. M., '14	++++	++++	++++	++++	++++	–
4. Cult., A. M., '15	++++	++++	++++	++++	++++	–
5. Cult., U. of M	++++	++++	++++	++++	++++	–
6. Cult., U. of C	++++	++++	++++	++++	++++	–
7. Sputums from 29 positive cases	++++	++++	++++	++++	++++	+++††
8. Sputum	++++	++++	++++	++++	++++	††
9. Urine	++++	++++	++++	++++	++++	††
10. Pus from cervical lymph glands	++++	++++	++++	++++	++++	++++
B. lepræ.						
1. Cult., A. M., '13	++++	++++	+++††	++++	+++††	
2. Cult., A. M., '14	++++	++++	+++††	++++	+++††	–
3. Cult., A. M., '15	++++	++++	+++††	++++	+++††	–
4. (2) Cult., Tulane	++++	++++	+++††	++++	+++††	–
B. smegmatis.						
1. Cult., A. M., '12	++++	++++	+++	++++	++	
2. Cult., A. M., '13	++++	++++	+++	++++	++	
3. Cult., A. M., '14	++++	++++	++††††	++++	+††	–
4. Cult., A. M., '15	++++	++++	++††	++++	++††	–
5. Cult. from normal smegma (a)	++++	++++	††	++	+††–	–
6. Normal smegma smears	Many acid-fast.	++++	††	++	+††	–
7. Cult. from normal smegma (b)	++++	+++	–––	††	††	–
8. Normal smegma smears (b)	Many acid-fast.	+++	–	††	+	–
9. Normal smegma smears (c)	A few acid-fast.	–	–	–	–	–
10. Normal smegma smears	A few acid-fast.	–	–	–	–	–
11. Normal smegma smears	Many acid-fast.	++++	††	+††	+††	–
12. Normal smegma smears	A few acid-fast.	††	–	–	–	–
13. (15) Normal smegma smears	None acid-fast.	–	–	–	–	–
B. Rabinovitch. (*Butter bacillus.*)						
1. Cult., A. M., '12	Many acid-fast.	++++	+++††	++++	+++	
2. Cult., A. M., '13	Many acid-fast.	++++	+++††	++++	+++	–
3. Cult., A. M., '14	Many acid-fast.	++++	+++††	++++	+++	–
Moeller's grass bacillus.						
1. Cult., A. M., '12	Many acid-fast.	++++	++††††	++++	+††	–
2. Cult., A. M., '13	Many acid-fast.	++++	+++	++++	++	–
3. Cult., A. M., '14	Many acid-fast.	++++	++††	++++	+††––	–
4. Cult., A. M., '15	Many acid-fast.	++++	++††	++++	+††	–

DISCUSSION.

It will be observed that by Fonte's method all of the members of the acid-fast group of organisms growing on culture media saprophytically were decolorized. This includes the pure cultures of the tubercle bacillus. None of the other methods would completely decolorize any of the members of this group of organisms, Bunge and Trantenroth's and Pappenheim's having as a rule a greater efficiency than Gabbet's or the Ziehl-Neelsen method, but even with these there were always some organisms of each member of the group retaining the fuchsin stain. On the other hand, in twenty-nine positive sputums from clinical cases of tuberculosis the tubercle bacilli remained acid-fast by Fonte's method as well as by the other methods. One sample of sputum from a doubtful case of tuberculosis (8), however, which when examined by Fonte's method showed only a few weakly acid-fast organisms containing the characteristic blue granules, appeared entirely different by the Ziehl-Neelsen and other methods, showing by these fifty and seventy-five strongly acid-fast organisms per field. This sputum was treated for a few minutes with 15 per cent antiformin, and after repeated washings the sediment was injected into guinea pigs. The injected sediment was rich in acid-fast organisms by the Ziehl-Neelsen method. After three months the pigs were normal in weight, did not respond to the tuberculin test, and at *post mortem* showed no evidence of tuberculosis except for the slight enlargement of a few mesenteric lymph glands. The tissue examination was negative. A similar report can be made in regard to urine sample (9), where many strongly acid-fast organisms were found by Gabbet's, Ziehl-Neelsen's, Pappenheim's, and Bunge and Trantenroth's methods, while only a few very weakly acid-fast organisms were in evidence by Fonte's method. Animal inoculation gave negative results. Clinically this case is not tuberculosis at present. In regard to the action of these two strains of acid-fast organisms one of the following must be true: (1) That the organisms are not tubercle bacilli; (2) that if they are they are so avirulent as not to cause tuberculosis in guinea pigs; (3) that the guinea pigs were unusually resistant to tubercle bacilli; (4) that the bacteria were attenuated or killed by the antiformin. In view of the fact that saprophytic acid-fast organisms have been isolated from sputums and urines, that guinea pigs are considered very susceptible to the tubercle bacillus, that the antiformin did not injure their acid-fast properties and is generally

considered not injurious to acid-fast tubercle bacilli,[16] and since the patients are not positively tuberculous clinically, it would appear that the organisms were probably saprophytes. The action of the organisms towards Fonte's method was a border-line one. In view of the work of Spengler[17] and also of Much[18] on non-acid-fast tubercle bacilli, one can not use this border-line reaction as evidence one way or the other.

It is hoped to continue this work and determine the relative frequency of occurrence of saprophytic acid-fast organisms in urine and sputums and to further check up the value of Fonte's and other promising methods of differentiation. If non-acid-fast or partially acid-fast tubercle bacilli were very commonly met with in urines and sputums from tuberculous patients it would be quite evident that no staining method based upon acid-fastness would be efficient, but there has not been much evidence brought forward to show anything but the scarcity of these non-acid-fast strains. The work of Wherry[19] indicates that this cause of acid-proofness of parasitic organisms is different from that of saprophytes. As to the acid-fast properties of intermediate organisms with a very low grade of virulence nothing has been reported along the line of Wherry's investigation. The many conflicting results which have led to the development of so many differential staining methods, added to conflicting opinions of the acid-proofness of different members of this group, suggests a wide variation in acid-proofness among the saprophytic members of the acid-fast group, at least. The work of this paper further confirms this fact. An explanation for the decolorization by Fonte's method of pure cultures of the tubercle bacillus, as well as other acid-fast organisms as reported in this paper, is that the tubercle bacillus, as well as the others, was growing saprophytically, hence its acid-proofness differed materially from that of the parasitic tubercle bacilli of the twenty-nine positive clinical cases of tuberculosis. These laboratory strains of the tubercle bacillus have also lost their pathogenicity. It may be that the groups are so closely related as to offer insurmountable obstacles to their absolute and complete differentiation. A method having a very high percentage of efficiency would be very valuable and desirable.

CONCLUSIONS.

1. That there is great variation in the acid-proofness of different strains of *B. smegmatis.*

2. That even in positive sputums there is some tinctorial difference of the tubercle bacillus toward Fonte's stain, whereas with the other methods very little if any tinctorial variations were observed.

3. That Gabbet's, Ziehl-Neelsen's, Pappenheim's, and Bunge and Trantenroth's methods are not at all reliable as a means of differentiating the tubercle bacillus from the rest of the acid-fast group.

4. That Fonte's method seems to be much superior to the other methods, but not entirely reliable in urine, and even in sputum examinations. The percentage of error can only be determined by much more extensive work.

5. That the error of all of these methods seems to be that of giving too many positive results.

6. That Fonte's method might prove quite serviceable in urine and feces examinations when used along with the clinical symptoms, cellular content and chemical tests. In case any of the latter are negative, animal inoculation should be resorted to. It must be remembered that none of the above staining methods can be relied upon alone. Probably few errors are made in sputum examination, on the positive side, by any of the routine methods, because clinical symptoms are usually pronounced before the sputum is examined. Perhaps many more sputums are called negative that should be positive than the reverse. However, there is at least some chance for error on the positive side in sputum examinations. With urine and feces the great problem confronting the bacteriologist is to rule out the saprophytes.

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NOTE.—For a discussion of the metabolism of the acid-fats group and confirmation of part of Wherry's work, see Kendall, J. Inf. Dis., Vol. 15, 1914. For the causes of acid-proofness, see Ritchie, J. Path. and Bact., 1905, 10, pp. 334, and Wills, Centralbl. f. Bakt. I, original, 1912, 61, p. 37.